

RESEARCH PAPER

Proteomics of *Thlaspi caerulescens* accessions and an inter-accession cross segregating for zinc accumulation

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Abstract

Metal hyperaccumulator plants have previously been characterized by transcriptomics, but reports on other profiling techniques are scarce. Protein profiles of *Thlaspi caerulescens* accessions La Calamine (LC) and Lellingen (LE) and lines derived from an LC×LE cross were examined here to determine the co-segregation of protein expression with the level of zinc (Zn) hyperaccumulation. Although hydrophobic proteins such as membrane transporters are not disclosed, this approach has the potential to reveal other proteins important for the Zn hyperaccumulation trait. Plants were exposed to metals. Proteins were separated using two-dimensional electrophoresis and those showing differences among accessions, lines or metal exposures were subjected to mass-spectrometric analysis for identification. Crossing decreased the number of different proteins in the lines compared with the parents, more so in the shoots than in the roots, but the frequencies of Zn-responsive proteins were about the same in the accessions and the selection lines. This supports the finding that the Zn accumulation traits are mainly determined by the root and that Zn accumulation itself is not the reason for the co-segregation. This study demonstrates that crossing accessions with contrasting Zn accumulation traits is a potent tool to investigate the mechanisms behind metal hyperaccumulation. Four tentatively identified root proteins showed co-segregation with high or low Zn accumulation: manganese superoxide dismutase, glutathione S-transferase, S-formyl glutathione hydrolase, and translation elongation factor 5A-2. However, these proteins may not be the direct determinants of Zn accumulation. The role of these and other tentatively identified proteins in Zn accumulation and tolerance is discussed.

Key words: Cross, hyperaccumulation, La Calamine, Lellingen, omics, proteomics, profiling, *Thlaspi caerulescens*, zinc (Zn).

Introduction

Some plants are adapted to environments with extremely high metal concentrations. A few of these plants, the hyperaccumulators, can effectively concentrate certain heavy metals in their shoots without signs of toxicity (Baker and Brooks, 1989). The molecular mechanisms of metal hyperaccumulation are far from being understood

(Verbruggen *et al.*, 2009). Since a number of applications in areas such as food and environmental quality could benefit from such knowledge, there is considerable interest in exploring the phenomenon.

The most intensively studied zinc hyperaccumulators are *Thlaspi caerulescens* and *Arabidopsis halleri*, both from the

Brassicaceae family (Verbruggen *et al.*, 2009). In these species, Zn hyperaccumulation and tolerance are, to a certain extent, constitutive at the species level (Assunção *et al.*, 2003a; Becher *et al.*, 2004). However, considerable heritable variation in degree exists among accessions, which opens up the possibility to identify the genetic determinants and molecular mechanisms underlying this variation through the analysis of intra-specific crosses. Besides the identification of quantitative trait loci for Zn accumulation (Assunção *et al.*, 2006; Deniau *et al.*, 2006), one important outcome from the analysis of the *T. caerulescens* intra-specific cross is that Zn accumulation and tolerance appear to be under independent genetic control (Assunção *et al.*, 2003b).

Hyperaccumulation of Zn includes enhanced rates of uptake into root cells, xylem loading/unloading, detoxification, and vacuolar sequestration in the leaves, as well as subsidiary characteristics, such as the improved homeostasis of other nutrients (e.g. Fe or Cu) and stress protection (Verbruggen *et al.*, 2009). At least some of these properties appear to be due to up-regulation of particular components of the Zn homeostasis machinery present in the non-hyperaccumulator species (Clemens *et al.*, 2002; Clemens, 2006; Broadley *et al.*, 2007; Krämer *et al.*, 2007). Transporters, including members of the ZIP, P-type ATPase, and CDF protein families are involved in Zn uptake into the root cells, xylem loading, and vacuolar sequestration, respectively (Verbruggen *et al.*, 2009). In addition, Zn-binding ligands such as malate or citrate (Salt *et al.*, 1999; Sarret *et al.*, 2002), as well as cell wall structure alterations, e.g. suberization/lignification of the tangential cell walls of the inner root cortical cell layer (Zelko *et al.*, 2008), have been suggested to contribute to hyperaccumulation. Recent studies emphasize the role of gene regulation and signalling in hyperaccumulation (Verbruggen *et al.*, 2009).

Broader molecular characterization of Zn hyperaccumulators has been performed mainly by transcriptomics. Cross-species comparisons have been reported between *Thlaspi caerulescens* and the non-accumulators *T. arvense* (Hammond *et al.*, 2006) and *Arabidopsis thaliana* (van de Mortel *et al.*, 2006, 2008), and between the metal-hyperaccumulating *A. halleri* and non-accumulating *A. thaliana* (Becher *et al.*, 2004; Weber *et al.*, 2004; Talke *et al.*, 2006) or *A. petraea* (Filatov *et al.*, 2006). Profiling has rarely been used to characterize Zn hyperaccumulators at the protein level (Tuomainen *et al.*, 2006). In proteomics, large and hydrophobic transporters and low-abundant or small polypeptides usually remain undetected in 2-DE-based proteomics. On the other hand, many other proteins, such as regulatory proteins and those contributing to stress protection that appear to have importance in Zn hyperaccumulation might be detected (van de Mortel *et al.*, 2006; Verbruggen *et al.*, 2009). In addition, post-transcriptional regulation could be very important, and there is often no proportionality between the transcript and protein abundance. Different profiling techniques are thus clearly complementary, and the proteomics approach is of increasing interest in exploring the hyperaccumulation phenomenon.

In our previous study, the proteomes of three *T. caerulescens* accessions from different origins were compared

(Tuomainen *et al.*, 2006). However, the genomes and proteomes of any accessions have been shaped and differentiated under the influence of many selective environmental factors other than those impacting on the degrees of metal accumulation and tolerance. Therefore, expression differences at the transcriptome or proteome levels are not necessarily causally related with the degrees of metal hyperaccumulation capacity or tolerance. To identify proteins putatively involved in Zn accumulation, the co-segregation of protein spot intensities and Zn accumulation capacity was investigated in a cross between plants from two *T. caerulescens* accessions LC and LE with contrasting degrees of Zn accumulation. The LC accession originates from a soil contaminated with calamine ore waste (Zn, Cd, Pb), and the LE accession from a non-metalliferous soil near Lellingen, Luxemburg (Meerts and Van Isacker, 1997). The LC accession is more tolerant to Zn and Cd, but has a lower Zn and Cd accumulation capacity than LE, when compared under controlled conditions at the same metal exposure levels, although LC shows much higher foliar concentrations under natural conditions (Assunção *et al.*, 2003a). Proteins that showed differences between the *T. caerulescens* accessions, between the lines derived from the cross, or between Zn exposures, were tentatively identified. Possible contributions of the proteins to the metal accumulation phenotype are discussed.

Materials and methods

Plants and metal exposures

Two *Thlaspi caerulescens* accessions were studied, La Calamine (LC) and Lellingen (LE), and five F₃ lines with contrasting Zn accumulation capacities, derived from a single F₁ LC×LE cross through selfing (Assunção *et al.*, 2003b; see Supplementary Figs S1 and S2 at *JXB* online). For the design of the experiment, see Supplementary Figs S3 and S4 at *JXB* online, as well. The LC and LE seeds were sown in a soil mixture of garden compost, peat, perlite, and sand (3:3:3:1 by vol.). After 28 d (LE) or 37 d (LC) the plants were in a 5–6-leaf stage. The roots were washed and the plants were placed in 800-ml pots for hydroponic culture. The medium was replaced three times during the first 2 weeks. After this preculture, the plants were exposed to the same nutrient solution, supplemented with 0, 2, 10, or 100 μM ZnSO₄ (Assunção *et al.*, 2003a). During the 5-d exposure, the nutrient solution was changed once. Shoot weights and root lengths were analysed, and the samples were frozen in liquid nitrogen and stored at –76 °C. Three low- and two high-Zn-accumulating F₃ lines of the LC×LE cross were grown in the same way (Assunção *et al.*, 2003a). The plants were exposed to 10 μM or 100 μM ZnSO₄ for 1 week, after which the shoots and roots were collected separately, frozen in liquid nitrogen, and stored at –80 °C.

Sampling for proteome analysis, protein extraction and 2-DE

For each line and metal exposure of the LC×LE cross, shoots or roots from 2–3 plants were pooled for protein extraction (see Supplementary Fig. S3 at *JXB* online). Three independent protein extracts were made from each pool, representing thus three technical replicates. The complete analysis set from the cross consisted of 30 shoot and 30 root samples (five lines, two exposures, three replicates). The analysis set of LC and LE accessions consisted of shoot and root samples derived from individual plants (see Supplementary Fig. S4 at *JXB* online). Each LE/LC accession–exposure combination consisted of three

samples, in total 24 samples from both shoots and roots (two accessions, four exposures, three replicates).

The protein extraction protocol is described by Koistinen *et al.* (2002). After washing and drying the protein pellets were dissolved in 2-DE sample buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, and 2% v/v Bio-Lyte 3/10 ampholyte (Bio-Rad, Hercules, CA, USA). Total protein concentrations were analysed using the Bio-Rad Protein Assay Dye reagent. The 2-DE was performed as described by Lehesranta *et al.* (2006). A total of 250 µg of shoot and 150 µg of root proteins were used per gel. After first dimension (isoelectric focusing, IEF), the strips (24 cm, pH 4–7 with linear range) were stored at –76 °C or subjected to equilibration in 6 M urea, 50 mM TRIS-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT for 15 min, and another 15 min in the same buffer in which DTT was replaced with 2.5% (w/v) iodoacetamide, followed by a second dimension with constant 20–25 mA current overnight. The gels were stained overnight with SYPRO Ruby fluorescence stain (Bio-Rad). The gels were washed with 10% methanol, 7% acetic acid for 1 h, and transferred in water. The gel images were acquired as described by Lehesranta *et al.* (2006).

Gel and data analysis

Gel image analysis was performed with PDQuest software version 7.1.1 (Bio-Rad). Protein spot intensities were normalized to minimize possible errors due to protein loading or staining intensity (the intensity of each spot in a gel was divided by the total intensity of all valid spots in the gel). The intensities of the missing spots were set as 0. In the analysis set of LC×LE cross, 2160 and 1470 spots for roots and shoots, respectively, were detected in at least three out of 30 gels. The intensities of the spots were analysed with Linear Mixed Model ANOVA in order to find differences between the F₃ lines with different metal accumulation capacity, between metal exposures and their interaction (*i.e.* protein spots with different intensities in different metal exposures among the lines). In the LC and LE accessions, a total of 2122 and 2319 spots for the roots and shoots, respectively, were detected in at least three out of 24 gels. The intensities of the spots were analysed with ANOVA to find proteins with differences between accessions, metal exposures or their interaction. *Post-hoc* comparisons were performed with Bonferroni multiple range test with $P < 0.05$. Statistical analyses were made using SPSS 14.0 software (SPSS Inc. Chicago, IL, USA). Effects of accessions, lines, Zn treatments and their interactions were considered to be significant at $P < 0.05$. Frequencies of spots with different intensities were compared between accessions and lines or roots and shoots with 2×2 contingency test, using Chi-square as a statistic.

In-gel digestion and mass-spectrometric analysis

The gels were silver-stained and mass-spectrometric samples were prepared as described by Lehesranta *et al.* (2005). For practical reasons, mass-spectrometric analysis was performed using one of three different systems (described in Supplementary data: 'Mass-spectrometric analysis', 'Parameters'). Databases used in MS/MS-searches (Yates *et al.*, 1995) for the identification with Analyst QS v1.1 and ProID v1.1 software were loaded from the NCBI (National Center for Biotechnology, non-redundant protein database, Viridiplantae, loaded 25 June 2008) and for Xcalibur Thermoquest (San Jose, CA, USA) with BioWorks 3.1 and Sequest algorithm (Sadygov *et al.*, 2004) from TAIR (The Arabidopsis Information Resource; TAIR7_pep, loaded 30 December 2007). *T. caerulescens* EST-database (Rigola *et al.*, 2006) was also used. For MASCOTSearch v1.6b13 script (<http://www.matrixscience.com>; Perkins *et al.*, 1999) NCBI nr Viridiplantae during 2008 was used.

Results

The parental *T. caerulescens* accessions LC and LE were compared with lines derived from LC×LE cross (see

Supplementary Figs S1, S2, S3, and S4 at JXB online) in order to find correlations between the Zn accumulation trait and expression levels of particular proteins. The plants, *i.e.* the parents and three low- and two high-Zn-accumulating inter-accession lines from the F₃ generation were exposed to Zn in hydroponics. To avoid toxicity, moderate metal concentrations and a rather short exposure were applied. After metal exposures, the plants appeared healthy; no significant differences in root length or shoot weight were observed between LC and LE plants exposed to different metal concentrations.

Soluble proteins from both shoots and roots were analysed using 2-DE. Since a direct comparison of the protein profiles of the parents and lines was technically rather difficult, these datasets were analysed independently. For the parental LC and LE accessions, differences between the accessions, metal exposures (0, 2, 10, and 100 µM Zn) and their interaction were analysed. For the lines derived from the inter-accession cross that showed differences in the metal accumulation trait, effects of metal exposures (10 µM and 100 µM Zn) and their interaction (*i.e.* proteins that responded differentially to metal exposures in different lines) were tested.

Table 1 shows the absolute and relative frequencies of spots significantly responding to plant origin (accessions or lines), Zn treatment, or the origin×treatment interaction. There was a dramatic drop in the number of spots differing between the high and low-accumulation lines, as compared with the parental accessions, but less so in the roots than in the shoots, both in the case of non-Zn-responsive and of Zn-responsive proteins (categories A/L and A+E1/L+E2) ($P < 0.001$ in a 2×2 Chi-square test). A comparable drop was

Table 1. Spots with significant differences in the analysis set of *T. caerulescens* LC and LE parental accessions analysed with ANOVA and low- and high-Zn accumulation phenotype of their inter-accession cross lines analysed with Linear Mixed Model ANOVA ($P < 0.05$) (see Supplementary Figs S1, S3, S4 at JXB online)

A, accessions; E1, exposures used for the accessions; A+E1, accessions and exposures; A×E1, accession×exposure interaction; L, accumulation phenotype; E2, exposures used for the inter-accession cross lines; L+E2, accumulation phenotype and exposures; L×E2, accumulation phenotype×exposure interaction. Spots with difference in A+E1 category are not included in categories A or E1 and spots with difference in L+E2 category are not included in categories L or E2.

	Number of significant spots	
	Shoots	Roots
LC and LE parental accessions		
A	539 (23.2%)	526 (24.8%)
E1	90 (3.9%)	41 (1.9%)
A+E1	43 (1.9%)	17 (0.8%)
A×E1	116 (5.0%)	108 (5.1%)
LC×LE inter-accession cross lines		
L	52 (3.5%)	155 (7.2%)
E2	40 (2.7%)	46 (2.1%)
L+E2	3 (0.2%)	15 (0.7%)
L×E2	27 (1.8%)	33 (1.5%)

observed for the proteins showing a significant treatment×accession/line interaction ($P<0.001$), but the relative decrease in spot number was similar in the roots and in the shoots. The fraction of proteins that responded exclusively to Zn exposure (categories E1 and E2) was not significantly different between the accessions and F₃ lines. The distribution of Zn-responsive proteins over shoots and roots was not significantly different between the accessions and lines. The spots subjected to mass spectrometry were selected based on significant differences ($P<0.05$) in the intensity. Approximately half of these spots were tentatively identified (Fig. 1; Tables 2–4; see Supplementary Tables S1–S4 and Supplementary data ‘Parameters’ at *JXB* online). ‘Tentative’ here means that the identity was not confirmed by functional analysis.

Proteins co-segregating with Zn accumulation in the lines and showing corresponding differential expression in the parents

Altogether nine proteins were identified from both parents and lines. Four tentatively identified root proteins showed

co-segregation with high or low Zn accumulation: manganese superoxide dismutase, glutathione *S*-transferase 16 (GST16), *S*-formyl glutathione hydrolase, and eukaryotic elongation factor 5A-2 (Figs 1, 2; Table 2). Only the level of glutathione *S*-transferase 16 was significantly affected by the Zn exposures used. No co-segregating shoot proteins were identified.

Glutathione *S*-transferase 16, *S*-formylglutathione hydrolase, and eukaryotic elongation factor 5A-2 were significantly higher in the LC accession and in the low-Zn-accumulating lines (Fig. 2; Table 2). No *S*-formylglutathione hydrolase spot was found in the root samples of LE and in the high-Zn-accumulating lines. This suggests that the level of the enzyme was either very low or the protein was sufficiently different to migrate differently in the 2-DE gels. In the shoots, the enzyme was found from both accessions but again the level was significantly higher in the LC accession. The level of manganese superoxide dismutase 1 (MSD1) was much higher in the LE accession and in both high-Zn-accumulating lines. The level of MSD1 was also high in one of the low-Zn-accumulating lines.

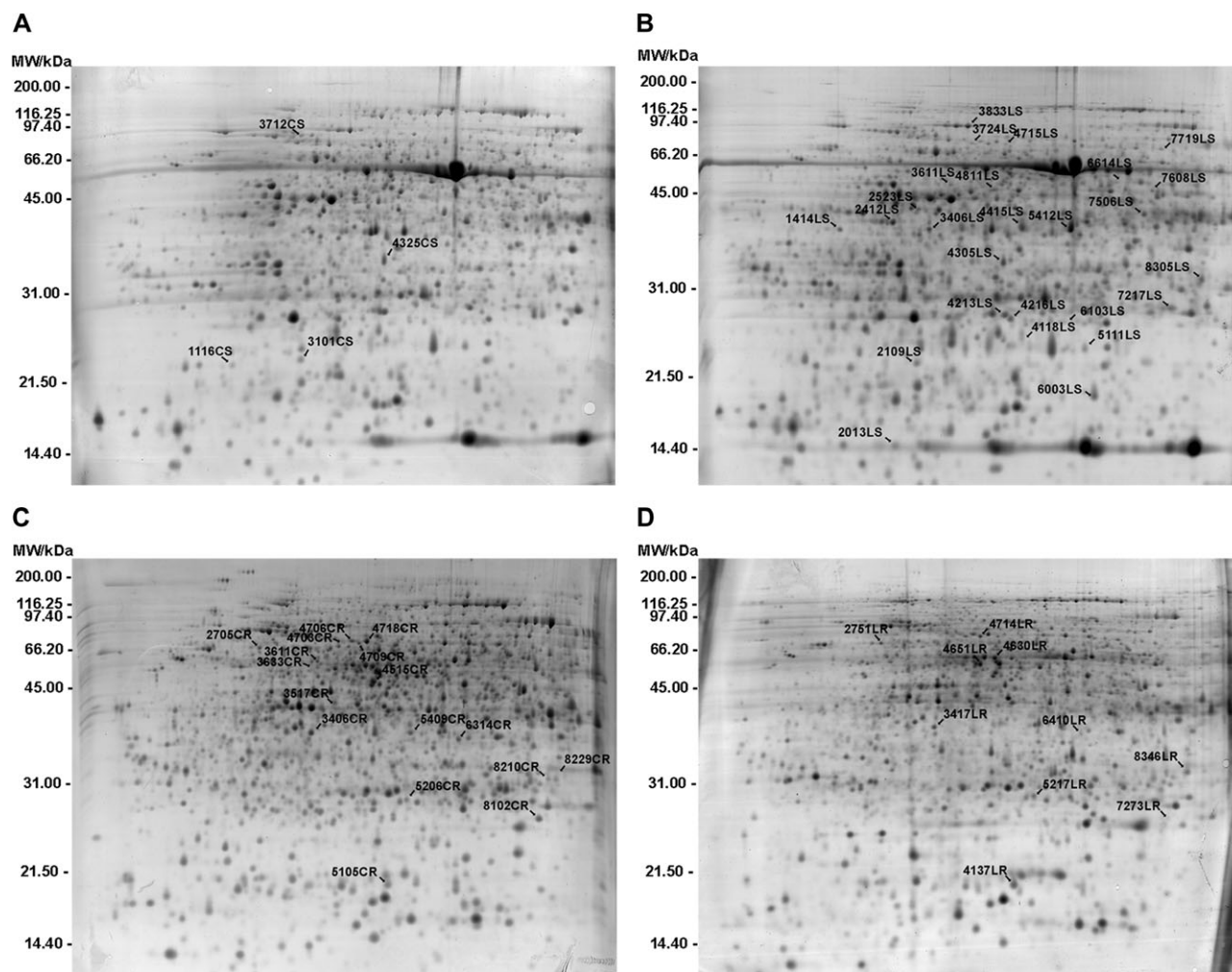


Fig. 1. Protein spots identified in shoots (A) and roots (C) of LC×LE inter-accession cross lines and shoots (B) and roots (D) of *T. caeruleus* LC/LE parental accessions. (Tables 2–4; see Supplementary Figs S1, S3, S4, and Supplementary Tables S1–S4 at *JXB* online).

Table 2. Tentatively identified shoot and root proteins from LC and LE parental accessions and lines from LC×LE cross (Figs 1, 2; see Supplementary Figs S1, S3, and S4 and Supplementary Tables S1–S4 at JXB online)

LR and LS, root and shoot protein, respectively, from parental accession LC or LE; CR and CS, root and shoot protein, respectively, from the cross; H, high-Zn accumulating-lines; L, low-Zn accumulating-lines.

Accessions (LC/LE)		Lines from cross (LC×LE)		Tentative identification ^c
Protein	Difference ($P<0.05$)	Protein	Difference ($P<0.05$)	
4137LR	LC>LE	5105CR	L>H	Eukaryotic elongation factor 5A-2 (At1g26630) ^a
5217LR	LC>LE	5206CR	L>H, 10 μ M Zn > 100 μ M Zn	Glutathione S-transferase 16 (At2g02930) ^a
7273LR	LE>LC	8102CR	H>L	Manganese superoxide dismutase (At3g10920)
8346LR	LC>LE	8229CR	L>H ($P>0.05$) ^b	S-formylglutathione hydrolase (At2g41530)
6410LR	–	6314CR	H>L	Annexin Arabidopsis 2 (At5g65020)
2109LS	LC>LE	3101CS	–	Thioredoxin-dependent peroxidase 1,2 (At1g65980, At1g65970) ^a , Peroxiredoxin type 2, putative (At1g60740) ^a
2751LR	LC>LE	2705CR	–	Protein disulphide isomerase 6 (At1g77510)
3417LR	LE>LC	3406CR	–	Isoflavone reductase, putative (At1g75280)
4305LS	LC>LE	4325CS	–	Glyoxalase I homologue (At1g11840)

^a Identification based on *T. caerulescens* EST. See Supplementary Tables S1, S2, and S4 at JXB online.

^b Non-parametrically tested, owing to zero values.

^c Homologue to *A. thaliana* is indicated in brackets.

Proteins showing incomplete correlation with Zn accumulation

Annexin Arabidopsis 2 (Table 2) was slightly but significantly higher in the roots of high-Zn-accumulating lines, but there was no significant difference between the parental accessions. Thioredoxin-dependent peroxidase (shoots), glyoxalase I (shoots) and protein disulphide isomerase (roots) were more highly expressed in LC than in the more Zn-sensitive LE accession, but no significant difference was found between the low- and high-Zn-accumulating lines. Isoflavone reductase was higher in the roots of LE than in LC accession but this was not reflected in the lines.

Proteins found either in the parents or lines

Several proteins that co-segregated with either high or low Zn accumulation were found from the lines but could not be identified from the parents (Table 3). This is probably explained by technical problems in matching the gels. In the shoot samples, the low-Zn-accumulating lines showed higher expression of heat shock protein 70 and copper homeostasis factor. In the root samples, most of the identified proteins also had higher expression in the low-Zn-accumulating lines: ThiJ-like protein, alanine aminotransferase 2 (AlaAT2), S-adenosylmethionine synthase 1 or 2, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, LOS2 (copper ion binding/phosphopyruvate hydratase), and malate dehydrogenase (MDH). Only the short-chain dehydrogenase/reductase (SDR) family protein showed higher expression in the high-Zn-accumulating lines.

A number of proteins were found that showed quantitative differences between LC and LE accessions or metal exposures (Table 4). As in the case of lines, the reason for

not finding the proteins from the cross might be explained by the difficulties in matching the gels. The strength of the findings in terms of linking them to hyperaccumulation phenotype is less than in the case of lines, since only two accessions were compared. Since the parental accessions also differ in their Zn tolerance, the proteins might as well be linked to the tolerance trait.

Discussion

To evaluate the results of transcriptomic studies in metal hyperaccumulators, proteomic studies are required but are barely available to date (Verbruggen *et al.*, 2009). In our previous study, the proteomes of roots and shoots of three *T. caerulescens* accessions (LC, LE and Monte Prinzera) exposed to excess Zn or Cd were compared (Tuomainen *et al.*, 2006). These accessions were collected from different geographic areas, suggesting that the proteomes are strongly affected by multiple environmental factors other than those exerting selective pressure on the metal accumulation and tolerance capacities. Therefore, it will be very difficult to sort out Zn hyperaccumulation-related proteins from proteome comparisons between different accessions. To reduce non-hyperaccumulation-related variation, the protein profiles of two accessions of *T. caerulescens*, LC and LE, with contrasting Zn accumulation and tolerance traits (Assunção *et al.*, 2003a; Meerts and Van Isacker, 1997), were compared with LC×LE F₃ lines selected for low and high Zn accumulation capacity, respectively (Assunção *et al.*, 2003b). In nutrient solution containing 10 μ M Zn the high-Zn-accumulating lines accumulated *c.* 10 times more Zn than did the low-Zn-accumulating lines (data not shown). Assunção *et al.* (2006) used the same cross for QTL analysis. The LC×LE F₃ lines were studied recently at the transcript level for their metallothionein gene expression

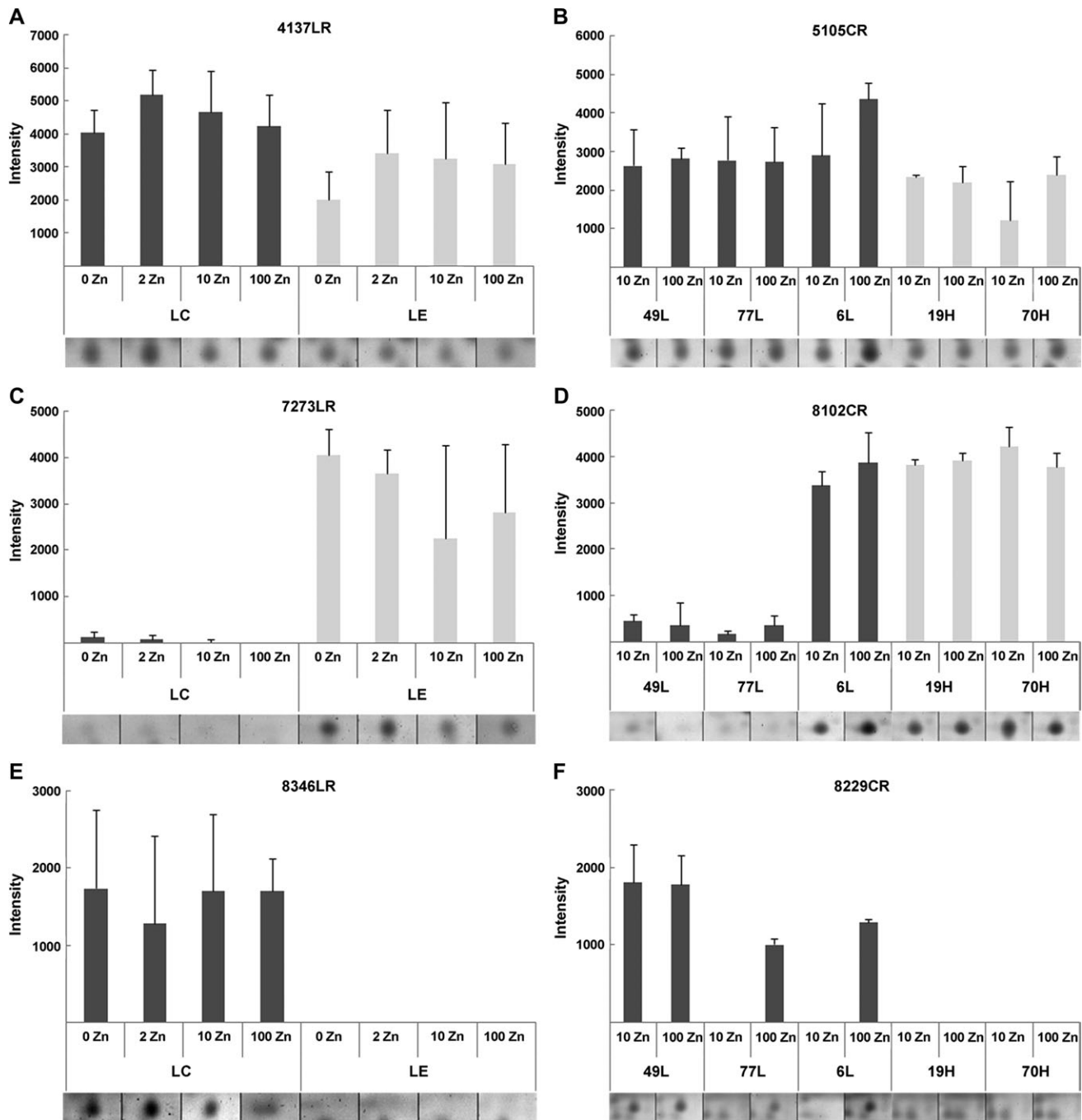


Fig. 2. Examples of root spots with different intensity among the *T. caerulea* parental accessions LC and LE and lines from inter-accession cross (49L, 77L, 6L; low-Zn-accumulating lines, 19H, 70H; high-Zn-accumulating lines). (A, B) Eukaryotic elongation factor 5A-2; (C, D) manganese superoxide dismutase 1; (E, F) S-formylglutathione hydrolase (Table 2; Fig. 1). Vertical bars represent mean values \pm SD of the spot in three gels. 0 Zn, 0 μ M ZnSO₄; 2 Zn, 2 μ M ZnSO₄; 10 Zn, 10 μ M ZnSO₄; 100 Zn, 100 μ M ZnSO₄.

(Hassinen *et al.*, 2009), but no proteomic data have been previously published on these lines.

The fraction of proteins differentially expressed between LC and LE was around five times higher than between the high- and low-Zn-accumulating lines, showing that the majority of differences unrelated to Zn accumulation was indeed eliminated by crossing (Table 1). The fraction of proteins, the level of which co-segregated with Zn accumulation in the lines was much higher in the roots than in the

shoots. This suggests that the difference in Zn accumulation between the lines is mainly determined by the roots. This is in agreement with the study of Guimarães *et al.* (2009) who showed that the scions of the non-hyperaccumulator *T. perfoliatum* hyperaccumulated Zn when grafted on a *T. caerulea* rootstock. Also HMA4 which is shown to be one of the determinants of the hyperaccumulation phenotype is mainly expressed in the roots (Hanikenne *et al.*, 2008). When expressed under the *A. halleri* promoter, it

Table 3. Tentatively identified shoot and root proteins from lines from LC×LE cross (Fig. 1; see Supplementary Figs S1 and S3 and Supplementary Tables S1 and S2 at JXB online) with significant difference between metal accumulation phenotypes and Zn exposures ($P<0.05$)

H, high-Zn accumulating lines; L, low-Zn accumulating lines; CR and CS root and shoot protein, respectively.

Protein	Difference ($P<0.05$)	Tentative identification ^c
1116CS	L>H	Copper chaperone (At3g56240) ^a
3712CS	L>H	Heat shock protein 70 (At1g56410, At5g02500, At3g09440, At5g02490)
3611CR	L>H	Alanine aminotransferase 2 (At1g72330)
3633CR	L>H ($P>0.05$) ^b	LOS2 (copper ion binding / phosphopyruvate hydratase) (At2g36530)
3517CR	L>H	ThiJ-like protein (Gi 33149230)
4703CR	L>H	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At1g09780 or At3g08590)
4706CR	L>H	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At1g09780)
4709CR	L>H	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At3g08590)
4515CR	L>H, 10 μ M Zn < 100 μ M Zn	S-adenosylmethionine synthase 1, 2 (At1g02500, At4g01850)
4718CR	L>H	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At3g08590)
5409CR	L>H	Malate dehydrogenase, MDH (At3g47520)
8210CR	H>L	Short-chain dehydrogenase/reductase (SDR) family protein (At3g03980)

^a Identification based on *T. caerulescens* EST. See Supplementary Tables S1 and S2 at JXB online.

^b Non-parametrically tested, owing to zero values.

^c Homologue to *A. thaliana* or other species is indicated in brackets.

enhanced metal accumulation in the shoots of *A. thaliana* (Hanikenne *et al.*, 2008). Furthermore, in *T. caerulescens* the concentration of histidine, which has been shown to contribute to metal (Zn and Ni) xylem loading, is enhanced in the roots but not in the shoots, compared to *T. arvense* (Richau *et al.*, 2009).

A relatively small fraction of the differentially expressed proteins was Zn-responsive. This is in line with our previous study (Tuomainen *et al.*, 2006). The Zn-responsive proteins were approximately equally represented among the accessions and lines, both in the roots and in the shoots. This would be expected if the Zn accumulation rates would be comparable between LC and the low-Zn-accumulating lines and between LE and the high-Zn-accumulating lines. It also confirms the absence of differential Zn-imposed stress levels among the accessions or lines.

Table 4. Tentatively identified shoot and root proteins from *T. caerulescens* LC and LE parental accessions (Fig. 1; see Supplementary Fig. S4 and Supplementary Tables S3 and S4 at JXB online) with differences between accessions and exposures ($P<0.05$)

LR and LS, root and shoot protein, respectively, from LC or LE.

Protein	Difference ($P<0.05$)	Tentative identification ^b
1414LS	LC>LE	Beta-1,3-glucanase 2 (At3g57260) ^a
2013LS	LC>LE	Aconitase C-terminal domain-containing protein (At2g43090 or At3g58990)
2412LS	LC>LE, 0 μ M Zn > 100 μ M Zn	Annexin Arabidopsis 1 (At1g35720)
2523LS	LC>LE, 10 μ M Zn > 2 μ M Zn, 100 μ M Zn	Adenosine kinase 2 (At5g03300)
3406LS	10 μ M Zn > 0 μ M Zn	Identical to pyruvate dehydrogenase E1 component subunit beta (At5g50850)
3611LS	LC>LE	ADP-glucose pyrophosphorylase small subunit (Gi 13487709)
3724LS	LC>LE, 10 μ M Zn > 2 μ M Zn	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At1g09780)
3833LS	LC>LE	Putative transketolase (At3g60750)
4213LS	LE>LC	Chaperonin 20 (At5g20720)
4216LS	LC>LE	Oxygen-evolving enhancer protein 2 (At1g06680) or Photosystem II subunit P-2 (At2g30790)
4415LS	LE>LC	Fructose-bisphosphate aldolase, putative (At2g21330)
4611LS	LE>LC	1-deoxy-d-xylulose 5-phosphate reductoisomerase (Gi 133753347)
4715LS	LC>LE, 10 μ M Zn > 2 μ M Zn	2,3-biphosphoglycerate-independent phosphoglycerate mutase (At3g08590)
5111LS	LC>LE	Lipocalin, putative (At5g58070)
5412LS	LC>LE	Fructose-bisphosphate aldolase (At4g38970 or At2g21330)
6003LS	LC>LE	Lipid-associated family protein (At2g22170)
6103LS	I; LE>LC (missing LC), LE; 0 μ M Zn, 2 μ M Zn, 10 μ M Zn > 100 μ M Zn	Glutathione S-transferase 1 (At1g02930), glutathione S-transferase 11 (At1g02920) or glutathione S-transferase tau 1 (At2g29450)
6614LS	LE>LC	3-Ketoacyl-acyl carrier protein synthase I (At5g46290)

Table 4. Continued

Protein	Difference ($P < 0.05$)	Tentative identification ^b
7217LS	LC>LE	Dehydroascorbate reductase (At1g75270 or Gi 76160951)
7506LS	LE>LC	Fructose-bisphosphate aldolase, putative (At4g26530)
7608LS	LE>LC	Citrate synthase 4 (At2g44350)
7719LS	LC>LE	NADP-malic enzyme 1, 2 or 3 (At2g19900, At5g11670 or At1g79750)
8305LS	LC>LE	S-formylglutathione hydrolase (At2g41530) ^a
4630LR	LC>LE	ATP synthase beta chain, putative, 1, 2 (At5g08680, At5g08670, At5g08690)
4651LR	LC>LE	ATP synthase CF1 alpha subunit (Gi 158938663)

^a Identification based on *T. caerulea* EST. See Supplementary Tables S3 and S4 at JXB online.
^b Homologue to *A. thaliana* or other species is indicated in brackets.

Theoretically, co-segregation of protein levels with the Zn-hyperaccumulation phenotype of the F₃ lines could be expected for the direct determinants of the phenotype, as well as for proteins that directly respond to the plant-internal Zn concentration. Quantitatively, the latter category would be expected to be much bigger, and of approximately equal size in the accessions and the F₃ lines, provided that the Zn accumulation levels are comparable between the parental accessions and the corresponding high- and low-Zn-accumulating lines. In agreement with this, the relative frequencies of Zn-responsive proteins were more or less similar in the accessions and in the lines. However, only one exclusively Zn-responsive protein (pyruvate dehydrogenase E1 component subunit beta) was tentatively identified in this study. Therefore, it remains a possibility that the composition of the Zn-responsive proteome may differ between the parental accessions and the lines, although this does not seem to be very likely. In addition, co-segregation of the expression level of a protein with Zn-accumulation capacity determinants may also result from linkage disequilibrium, or from direct or indirect coupling at the functional, physiological level. Differential protein expression between the parental accessions is not necessarily a prerequisite for co-segregation with the Zn accumulation phenotypes of the F₃ lines. If both parents contribute trait-enhancing as well as trait-suppressing alleles, which has been shown to be the case in this and other *T. caerulea* crosses (Assunção *et al.*, 2006; Deniau *et al.*, 2006), then protein expression might transgressively segregate if it is under the control of multiple QTLs. In fact, in the study of Assunção *et al.* (2006) two QTLs were found for Zn accumulation in the roots. Therefore, it is important to consider proteins that are differentially expressed exclusively among the lines.

Proteins co-segregating with Zn accumulation in the lines and showing corresponding differential expression in the parents

Manganese superoxide dismutase: A protein tentatively identified as manganese superoxide dismutase 1 (homologous to At3g10920; MSD1) showed higher intensity in the roots of LE accession compared to LC. The intensity of the protein spot was also high in high-Zn-accumulating LC×LE lines and low in two low-Zn-accumulating LC×LE lines whereas the third low-Zn-accumulating line had a level similar to the high-Zn-accumulating lines (Table 2; Figs 1, 2). MSD1, related to ROS removal, is the primary protective enzyme against oxidative stress in mitochondria. Silencing of MSD1 leads to reduced root growth in *A. thaliana*, affecting the redox homeostasis in mitochondria, while not disturbing the overall redox balance of the plants (Morgan *et al.*, 2008). However, MSD1 is certainly not a direct determinant of high Zn accumulation, because one of the low-Zn-accumulating lines showed the same high expression level as found in the high-Zn-accumulating lines and LE (Fig. 2). This suggests that the (partial) co-segregation of high MSD1 expression with the high-Zn-accumulating phenotype may be caused by genetic linkage, and that a crossover might have been taken place in one of the low-Zn-accumulating lines. The intensity of the MSD1 spot in the LC accession and the two low-Zn-accumulating lines was very low, which might indicate that most of the protein was post-translationally modified and thus differently located in the 2-DE map. However, there are no indications of any post-translational modifications of this protein.

Glutathione S-transferase 16: A protein tentatively identified as glutathione S-transferase 16 (homologous to At2g02930; GST16) showed significantly higher intensity in the roots of the LC accession and the low-Zn-accumulating LC×LE lines as compared to LE and the high-Zn-accumulating lines (Table 2). GSTs belong to a multigene family with six subclasses (Marrs *et al.*, 1996; Dixon *et al.*, 2002). They function, for example, by detoxifying endogenous and exogenous xenobiotics such as metals, ROS, and ROS-induced lipid peroxidation products by glutathione, or may function as peroxidases (Dixon *et al.*, 2002, 2009). They are known to respond to various stimuli, for example, to compounds such as salicylic acid and H₂O₂ (Sappl *et al.*, 2009), as well as to several environmental factors like metals (Roth *et al.*, 2006; Sarry *et al.*, 2006; Ahsan *et al.*, 2008; van de Mortel *et al.*, 2008). Hammond *et al.* (2006) observed higher expression of the same gene in *T. caerulea* than in *T. arvense* although in the shoots. Remarkably, in our study, higher level of the protein co-segregated with low-Zn-accumulation capacity. Because the specific function of this protein is unknown, we cannot explain this pattern of co-segregation.

S-formylglutathione hydrolase: S-formylglutathione hydrolase (homologous to At2g41530) was higher in the roots of

the low-Zn-accumulating lines and in both roots and shoots of the LC accession (Fig. 2), thus following the low-Zn-accumulation trait. No *S*-formylglutathione hydrolase spot was found in the root samples of LE and the high-accumulating lines. This suggests that the level of the enzyme was either very low or the protein was sufficiently different to migrate differently in the 2-DE gels, for example, due to post-translational modification. In the shoots, the protein spot was found in both accessions, albeit at a higher level in the LC accession, suggesting that the primary structure of the protein is similar in both accessions. The enzyme produces glutathione and formate from toxic formaldehyde generated, for example, in the pectin demethylation, from protein repair and oxidative demethylation reactions (Fall and Benson, 1996; reviewed in Igamberdiev *et al.*, 1999). Pectin demethylation creates free carboxyl groups (Pelloux *et al.*, 2007) which function as binding sites for metals and may contribute to the metal accumulation capacity. However, *S*-formylglutathione hydrolase is not responsible for the demethylation itself and it co-segregates with low Zn accumulation in this experiment, showing that it is not a primary determinant of Zn accumulation capacity.

Eukaryotic elongation factor 5A-2: Eukaryotic elongation factor 5A-2 (homologous to At1g26630) was higher in the roots of the low-Zn-accumulating lines and of the LC accession (Table 2; Figs 1, 2), thus following the low-Zn-accumulation trait. Eukaryotic elongation factor 5A-2 has a fundamental role in growth and development (cell proliferation, cell growth, and cell differentiation), as well as in senescence-type programmed cell death (Feng *et al.*, 2007). This protein may have a role in the regulation but the finding cannot be explained in terms of Zn accumulation.

Proteins co-segregating with Zn accumulation but not identified or found to be differentially expressed in the parents

None of the proteins that showed higher intensity in the low-Zn-accumulating lines are expected to be primary determinants of Zn accumulation capacity (Table 3). Some of the proteins may, however, be related to metal-associated metabolism. These include *S*-adenosyl methionine synthase 1 or 2 (homologous to At1g02500 or At4g01850) involved in the synthesis of nicotianamine which is important as a chelator of Fe, Co, Ni, and Zn. ThiJ-like protein (homologous to Gi 33149230) participates in the biosynthesis of thiamine that has been suggested to have a role in the protection of plant cells against oxidative stress (Tunc-Ozdemir *et al.*, 2009). Alanine aminotransferase 2 (AlaAT2, homologous to At1g72330) has a role in alanine metabolism. The content of alanine has been reported to increase in hypoxia, where up-regulation of alanine aminotransferases may help to convert alanine to be used as a nitrogen source and to maintain the carbon–nitrogen balance during the recovery from the stress (Allan *et al.*, 2008; Miyashita *et al.*, 2007). Also other abiotic stresses like cold and drought increase alanine concentrations,

which is supposed to be a consequence of a changed redox balance (Allan *et al.*, 2008). ThiJ-like protein and AlaAT2 identified from the inter-accession cross may have protective roles in stress. Proteins with higher intensity in the high-Zn-accumulating lines were short-chain dehydrogenase/reductase family protein (homologous to At3g03980) with unknown function and annexin Arabidopsis 2 (homologous to At5g65020). Annexins belong to a multigene family of plasmamembrane and endomembrane binding proteins through Ca^{2+} -dependent or -independent manner (reviewed in Mortimer *et al.*, 2008). Annexins have been suggested to function as, for example, GTPases (Shin and Brown, 1999), sensors, peroxidases, and channels (Laohavisit *et al.*, 2009). Since annexins are targets for Ca^{2+} (reviewed in Mortimer *et al.*, 2008), an important molecule in cell signalling, they may have a role in stress response.

Proteins with different expression in parents but not co-segregating with Zn accumulation in the lines

Four proteins identified both from the parents and lines were differentially expressed in the parents, but they showed no co-segregation with Zn-accumulation capacity (Table 2). Since the lines were not selected for tolerance but the parents differ in their Zn tolerance, those proteins that show differences between the parents may be linked to the Zn tolerance trait but also to any other trait that differs between the parents. Thioredoxin-dependent peroxidase (homologous to At1g65980 or At1g65970 or At1g60740), glyoxalase I (homologous to At1g11840) in the shoots and protein disulphide isomerase (homologous to At1g77510) in the roots were more highly expressed in the LC than in the more Zn-sensitive LE accession. These proteins are not primary determinants of Zn accumulation. However, some of them may be involved in metal-related processes. Thioredoxin-dependent peroxidase could be involved in lignification, that is suggested to be a common response to excessive metal exposure (Ederli *et al.*, 2004; van de Mortel *et al.*, 2008). Protein disulphide isomerase belongs to the thioredoxin superfamily and could be involved in the regeneration of oxidized proteins or in the reduction of glutathione conjugates, compounds that may have been produced due to changed intracellular redox status induced by, for example, metal exposures. Overexpression of *Brassica juncea* glyoxalase I caused higher tolerance to Zn in tobacco (Singla-Pareek *et al.*, 2006) possibly through the regeneration of glutathione. However, *A. thaliana* T-DNA insertion mutants with 1–3% of the transcript level compared to the wild type were not significantly compromised for Zn tolerance or Zn accumulation behaviour, which argues against the role for glyoxalase I in Zn tolerance (MH Tuomainen *et al.*, unpublished results). Isoflavone reductase from flavonoid metabolism in the roots was higher in the roots of LE than in LC. Flavonoids have been found from root exudates of plants growing on metal-enriched soil and they have been suggested to play a role in processes of adaptation to metal-contaminated soil as chelating agents for

poorly soluble mineral nutrients (Quartacci *et al.*, 2009). This could also be the case in our study.

Proteins identified only from the LC and LE parents

Because these proteins were not identified in the lines, a connection with the Zn accumulation traits cannot be established. This does not exclude the possibility that some of the proteins could be involved in Zn accumulation. However, clear candidates were not apparent. Citrate synthase (Table 4) might contribute to Zn sequestration in the shoots and, therefore, potentially to Zn accumulation. However, the accumulation capacity is determined by the roots rather than by the shoots, which argues against the role for citrate synthase in Zn accumulation (see above). Proteins that are more highly expressed in LC than LE might play a role in Zn tolerance. Some of these proteins may be involved in defence against oxidative stress, such as dehydroascorbate reductase and possibly lipocalin (Charron *et al.*, 2008) and a glutathione *S*-transferase-family protein (Sappl *et al.*, 2009). Adenosine kinase 2 (ADK) and glucan 1,3-beta-glucosidase showed higher expression in LC than in LE. Adenosine kinase is involved in methyl recycling and, for example, pectin methyl esterification affecting cell wall integrity (Pereira *et al.*, 2006) and beta-1,3-glucanase (pathogenesis-related protein) is a cell wall remodelling enzyme from cellulose biosynthesis pathway. Cell structure may be important in protecting plants from metal excess (Ederli *et al.*, 2004; van de Mortel *et al.*, 2008) and therefore the proteins in the metabolic route may be important in metal tolerance. A few of the proteins have been previously found to be differentially expressed at the mRNA-level in Zn-accumulating and non-accumulating plants, such as fructose biphosphate aldolase between *A. thaliana* and *T. caerulescens* (van de Mortel *et al.*, 2006), and OEE2 between *T. arvense* and *T. caerulescens* (Hammond *et al.*, 2006). It is not known whether they play a role in metal-related processes.

Conclusions

In conclusion, our study shows that crossing and selection for Zn accumulation capacity indeed eliminates a major part of the proteomic variation that exists between different *T. caerulescens* accessions, suggesting that much of the latter variation is unrelated to the variation in Zn accumulation capacity. Of the proteins, the expression of which co-segregates with Zn accumulation among the lines selected from the cross, a great majority was differentially expressed in the roots, suggesting that the Zn accumulation capacity is determined by characteristics of the root rather than the shoot, in agreement with previous studies. However, very few of the proteins found to be differentially expressed between the low- and high-Zn-accumulating lines seem to be potentially involved in Zn accumulation, and the reasons for their co-segregation with the Zn accumulation phenotype remain elusive. Physical genetic linkage of the encoding or *cis*-regulatory

sequences with the direct genetic determinants of the Zn accumulation phenotype does not seem to explain the majority of the cases of co-segregation, since this does not explain their strong over-representation in the root proteome. Inter-dependent trans-regulation is much more likely, although any circumstantial evidence of this has been lacking throughout. The observation that the relative frequencies of Zn-responsive proteins were about the same in the accessions and the selection lines suggests that Zn accumulation itself is not the reason for the co-segregation. However, the possibility remains that the identity of the Zn-responsive proteins might differ between accessions and F₃ lines. Finally, the apparent lack of clear-cut Zn-accumulation-related proteins among the differentially expressed proteins might well be due to the low identification rate. To understand better the intra-specific variation in Zn hyperaccumulation capacity within *T. caerulescens* at the proteomic level, further sequence information on this species is urgently required.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Origin of the plants used for proteomic analysis. The information is derived from Assunção *et al.* (2003b).

Supplementary Fig. S2. Zn tolerance of *T. caerulescens* accessions (LC and LE) and inter-accession cross lines (19H, 70H, 49L, 77L, 6L). The data are derived from Assunção *et al.* (2003b).

Supplementary Fig. S3. Flow chart of the experiments performed with *T. caerulescens* inter-accession cross lines.

Supplementary Fig. S4. Flow chart of the experiments performed with *T. caerulescens* accessions.

Supplementary data: Mass-spectrometric analysis. Methodology for mass-spectrometric analysis of the protein spots.

Supplementary data: Parameters. Parameters used in database search for the peptides found (Supplementary Tables S1–S4).

Supplementary Table S1. Tentatively identified shoot proteins from *T. caerulescens* LC×LE cross lines (19H, 70H, 49L, 77L, 6L; Supplementary Figs S1 and S3).

Supplementary Table S2. Tentatively identified root proteins from *T. caerulescens* LC×LE cross lines (19H, 70H, 49L, 77L, 6L; Supplementary Figs S1 and S3).

Supplementary Table S3. Tentatively identified shoot proteins from *T. caerulescens* LC/LE accessions (Supplementary Fig. S4).

Supplementary Table S4. Tentatively identified root proteins from *T. caerulescens* LC/LE accessions (Supplementary Fig. S4).

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